Hydrogen sulphide plays a role in the development of hepatopulmonary syndrome

A dissertation submitted in partial fulfilment of the requirements for DM (Hepatology) examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai, to be held in August 2014.

Certificate

This is to certify that the dissertation entitled **"Hydrogen sulphide plays a role in the development of hepatopulmonary syndrome"** is a bonafide work done by Dr. Chinmay Bera in partial fulfillment of the university rules and regulations for award of DM (Hepatology) under my guidance and supervision during the academic year 2014.

Name & signature of the Guide:

Prof. CE.Eapen, MD, DM,
Professor and Head,
Department of Clinical Gastroenterology and Hepatology,
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November 2, 2012

Dr. Chinmay Bera
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Sub: **Fluid Research grant project NEW PROPOSAL:**
To study the incidence, natural history and significance of hydrogen sulfide in hepatopulmonary syndrome.
Dr. Chinmay Bera, Senior Registrar, Hepatology, Dr. CE Eapen, Dr. Ashish Goel, Dr. Jeyamani R, Hepatology, Dr. Purendra Kumar Pati, Cardiology, Dr. Anup Ramachandran, Ms. Kavitha Rajathi, The Wellcome Trust Research Laboratory,Dr. K A. Balasubramanian.

Ref: IRB Min. No. 7861 dated 04.06.2012

Dear Dr. Chinmay Bera,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "To study the incidence, natural history and significance of hydrogen sulfide in hepatopulmonary syndrome" on June 4, 2012.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Information Sheet and Informed Consent Form (English)
3. Cvs of Drs. Chinmay Bera, CE Eapen, Ashish Goel, Jeyamani R, Anup Ramachandran, K A. Balasubramanian.Ms. Kavitha Rajathi,
4. A CD containing documents 1 – 3

The following Ethics & Research Committee members were present at the meeting held on 4th June 2012, Monday by 1.30 PM in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

A sum of Rs 40,000/- (Rupees Forty thousand only) will be sanctioned for 12 months. A subsequent installments of 40,000/- each will be released at the end of the first year following the receipt of the progress report (Total amount 80,000/-).

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
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CC: Dr. CE Eapen, Professor, Department of Hepatology, CMC

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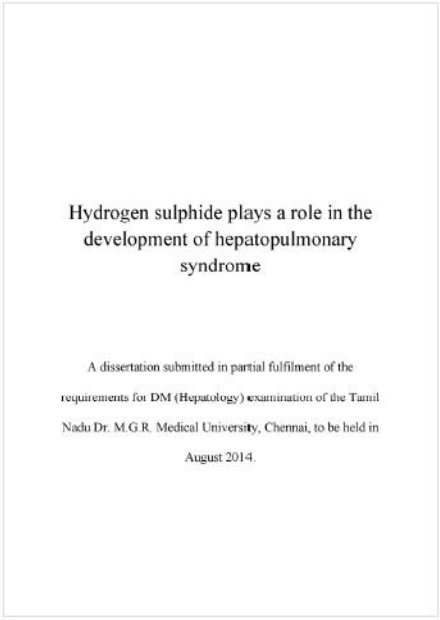

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Acknowledgement

I take this opportunity to express my sincere gratitude to my guide, Dr CE. Eapen, Professor and Head, Department of Hepatology, for his guidance, encouragement and constant support in undertaking and completing this project. I express my sincere thanks to Dr KA Balasubramaniam and Dr Anup Ramachandran, Professors, Department of gastrointestinal sciences for their constant support during the project as well as for doing hydrogen sulphide estimation in their laboratory. I express my sincere thanks to Dr. Purnendu kumar pati, Professor, Department of Cardiology, for his constatnt support and doing the contrast echocardiography. I take this opportunity to thank Mrs. Kavitha Reuben, Wellcome trust laboratory, who took great efforts to do hydrogen sulphide estimation in the laboratory.

I take pleasure in thanking Dr. Ashish Goel, Department of Hepatology who supported me throughout the course and helped me in the statistical analysis. I also thank all consultants of our department, all my co registrars and colleagues for their help and support during the study period. I also thank fluid research grant, Christian Medical College, Vellore, for the financial support.

CONTENTS

INTRODUCTION

Hepatopulmonary syndrome (HPS) is an uncommon complication of chronic liver disease and portal hypertension. It is characterised increased alveolar arteriolar oxygen gradient leading to hypoxemia in patients with portal hypertension with or without chronic liver disease. Patients with intrapulmonary vasodilatation (IPVD) as manifested by increased alveolar arteriolar oxygen differences do not always have hypoxemia known to have subclinical HPS. HPS do not always depend on the underlying liver disease severity. HPS have been described in patients with abnormal porto-caval connection without any evidence of chronic liver disease.

Exact pathogenesis is still not understood. Multiple factors have been proposed as pathogenesis of HPS in the development of intrapulmonary vasodilatation (IPVD) and hypoxemia. Vasodilatory gaseous transmitters like nitric oxide (NO) and carbon monoxide (CO) known to play important role in the development of IPVD. Along with the Vasodilatory properties of NO, its role in the neo angiogenesis and formation intrapulmonary shunt also has been proposed.

Hydrogen sulphide, originally known as environmental pollutant, most recently recognised as a blood flow regulator and mucosal healing properties. HS is synthesized from cysteine by different enzymes in different places. It has been studied in patients with circulatory failure and in patients with inflammatory

bowel disease for its mucosal healing properties. Its role in regulation of sinusoidal blood flow has been studies by Fiorucci et al. There is no study regarding its role in pathogenesis of hepatopulmonary syndrome.

REVIEW OF LITERATURE

Hepatopulmonary syndrome:

Hepatopulmonary syndrome is a lung disorder usually found in patients with liver diseases, was first described long back in 1966 by Berthelot et al(1). Marked pulmonary vascular dilatation was noted in autopsy specimen of patients with cirrhosis(2). The term HPS was first coined in 1977(3).

Reported incidence of HPS varies in different studies. It also depends on the cut off value of hypoxia taken into consideration(4). 10 to 30% prevalence have been reported from the liver transplant centre(5). In a study from India by Gupta et al, prevalence of HPS was much higher in patients with chronic liver disease than extra hepatic portal venous obstruction(6). The cut off for partial pressure of oxygenation and alveolar-arterial oxygen gradient, as recommended by a task force is mention below in the table.

Degree of severity	
Mild	Alveolar-arterial oxygen gradient ≥15 mm Hg, partial pressure of oxygen ≥80 mm Hg
Moderate	Alveolar-arterial oxygen gradient ≥15 mm Hg, partial pressure of oxygen ≥60 to <80 mm Hg
Severe	Alveolar–arterial oxygen gradient ≥15 mm Hg, partial pressure of oxygen ≥50 to <60 mm Hg
Very severe	Alveolar-arterial oxygen gradient ≥15 mm Hg, partial pressure of oxygen <50 mm Hg

Table 1. Diagnostic Criteria for the Hepatopulmonary Syndrome.*

*****Adopted from ref. No 14.

Screening for hepatopulmonary syndrome:

Liver transplantation is the only effective treatment available in patients with HPS. Severity of hypoxemia also affects the mortality of patient both preoperatively and post operatively. Severity of hypoxemia also does not depend on the severity of liver disease. The American Association for the Study of Liver Disease Practice Guidelines recommend early referral and evaluation for liver transplantation in patients with HPS(7). The United Network for Organ

Sharing (UNOS) liver allocation policy also supports Model for End-stage Liver Disease (MELD) exception points to provide a patient a reasonable probability of being transplanted within 3 months in patients with severe HPS that is the partial pressure of oxygen (PaO_2) is 60 mm Hg on room air(8).

This is important as patients with hepato pulmonary syndrome may have very low MELD score and they may not be eligible for listing in the cadaveric listing programme. This signifies the importance of early detection and referral along with extra point allocation to the patients with HPS for liver transplantation. Thus, identifying these patients has clinical implications. Screening of those groups of patients is not always cost effective as there are no specific clinical findings to suggest HPS in patients with chronic liver disease. In a study by Roberts et al describe the mode of HPS screening and cost effectiveness of the same in candidates listed for liver transplantation. Author concluded that selective group of patients with HPS especially those are being listed for liver transplantation can be screened by pulse oximetry. prevalence of HPS is also important in deciding the utility of screening programme (9).

Clinical manifestations:

This varies from asymptomatic to oxygen dependant. Clinical features are of limited use in predicting HPS. Dyspnoea at rest or exertional dyspnoea may be

the presenting symptoms. In patients with chronic liver disease dyspnoea may be due to multiple factors like: anaemia, tense ascites, pleural effusion or HPS. Few clinical signs like spider naevi, clubbing and cyanosis have been associated with HPS. However presence of orthodeoxia in a patient with chronic liver disease strongly suggests HPS. Orthodeoxia can be measured by arterial blood gas analysis. 5% or more drop in the PaO_2 in arterial blood when patient moves from the supine to standing position. Patient may describe it as platypnoea(10).

Natural history:

Median survival of patients with HPS is considerably lower than the age, sex, CTP and MELD matched cohort without liver disease. Median survival of patient not undergoing liver transplantation is around 24 months as compared to 87 months without HPS(11). Similarly 5 years survival rate also significantly decreased in HPS patients compared to those without HPS (63% Vs 23%)(12). Worse survival has been observed in severely hypoxemic that in partial pressure of oxygen <50%, which suggest the fact that progressive deterioration of liver function due to relative ischemia of the liver(13). Although the death in patients with HPS hardly attributed by worsening liver function rather than due to hypoxemia.

Pathogenesis:

Three mechanisms have been described in the pathogenesis of HPS(14).

1) Ventilation perfusion mismatch
2) Right to left shunting
3) Limitation in diffusion.

Dilatation of pre-capillary and capillary vessels and increase the numbers of dilated vessels leading to right to left shunting and ventilation perfusion mismatch. Various gaseous mediators are responsible for the mechanism(15).

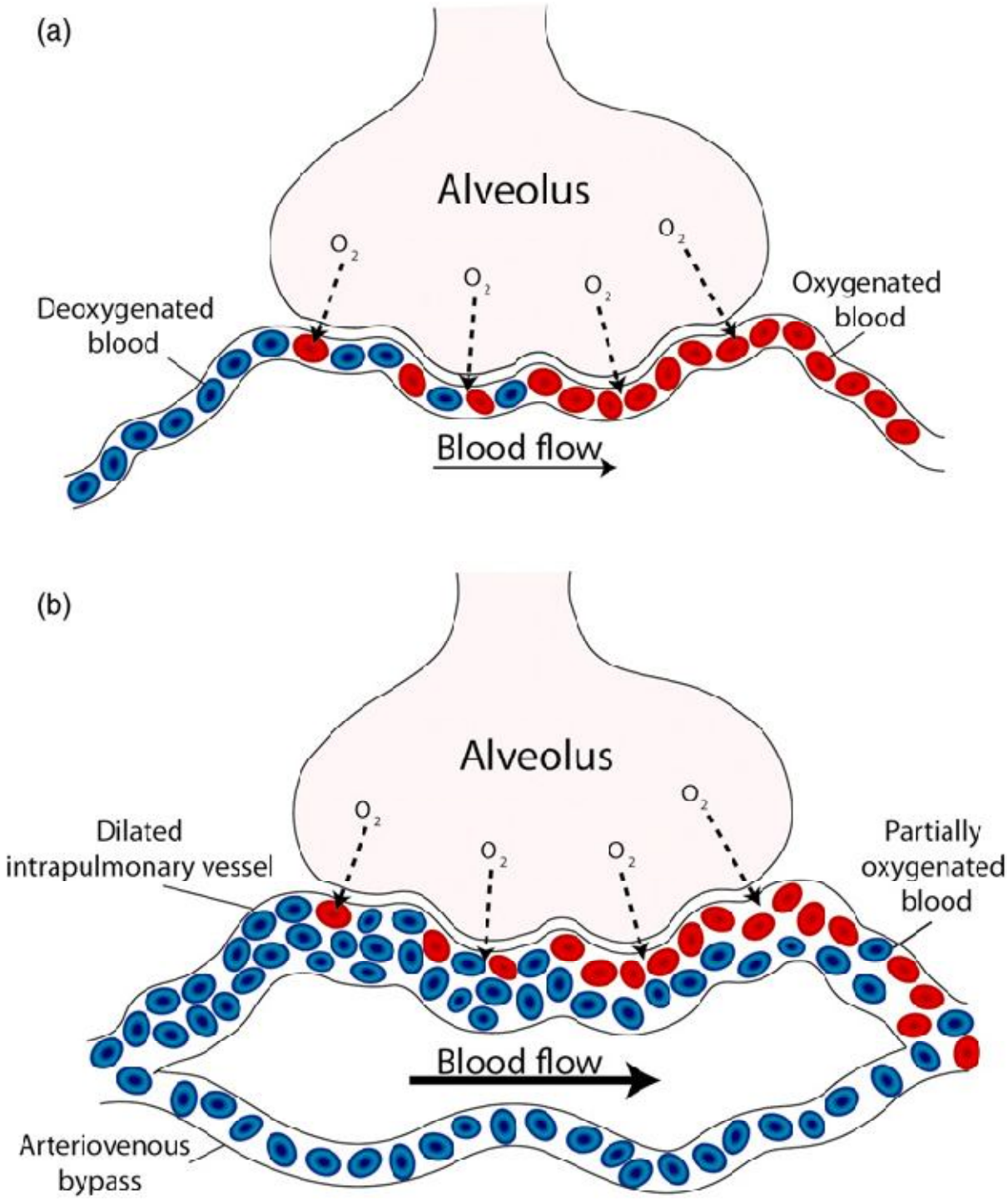


Figure 1: The pathophysiology of hepatopulmonary syndrome. (a) Represents normal alveoli, where oxygen diffuses from a normal alveolus into a pulmonary capillary. (b) Represents the exchange of gas in hepatopulmonary syndrome

Nitric oxide:

Nitric oxide (NO) is synthesized by nitric oxide synthase (NOS) from L-arginine. NO is considered as a relaxant to vascular smooth muscle cell and a potent vasodilator(16). The role of NO in the development of HPS has been studied extensively. In a study by... increase level of exhaled NO seen in patients with cirrhosis with HPS as compared to patients without HPS(17).

Pulmonary angiogenesis:

Along with dilatation of the pre-capillary and capillary vessels there is also proliferation of the new vessels around the alveolus. Increase in the TNF-α results in recruitment of pulmonary intravascular monocyte and activation of VEGF (vascular endothelial growth factor) dependent pathways are thought to be responsible for this(18).

Endothelin 1(ET-1):

Plasma level of ET-1 is increased in patient with HPS as compared to patients without HPS(19). Overexpression of endothelin B receptors have been noted and activation of ETb receptors result in NO induced vasodilatation(20).

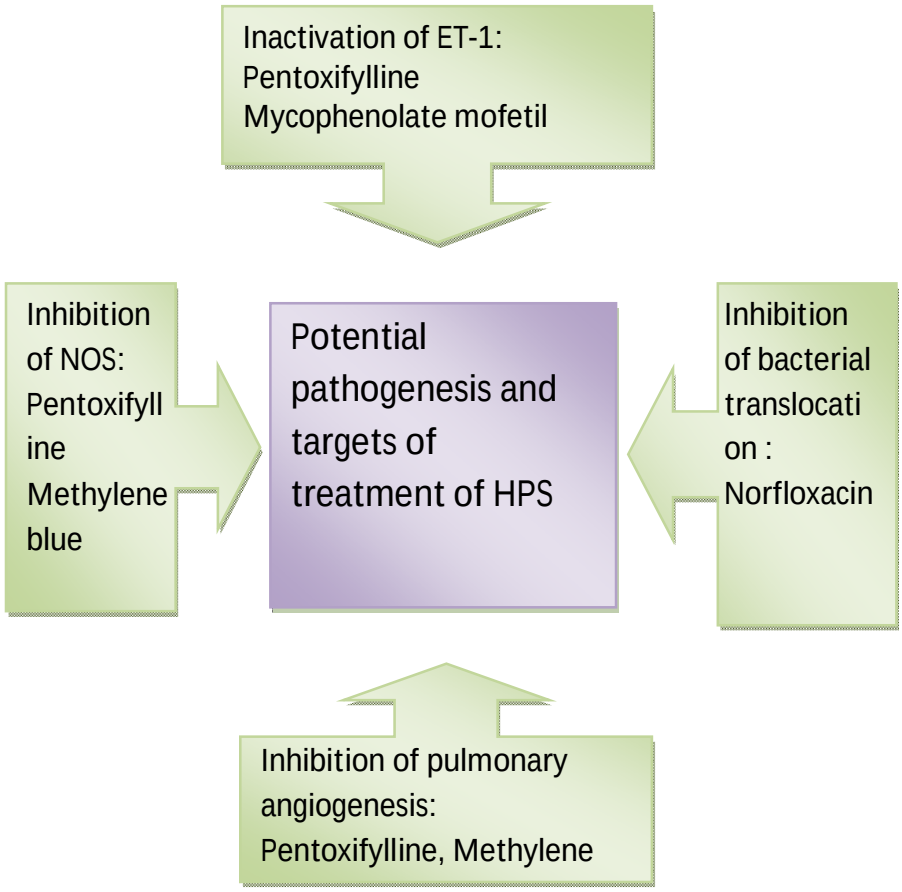


Figure 2: Possible mechanism and therapeutic options in hepatopulmonary syndrome

No appears to be key mediators of vasodilatation in the development of hepatopulmonary syndrome. In experimental HPS increase in pulmonary endothelin B receptors were noted in pulmonary circulation. Reasons for the increase in this receptos are not well understood. This may be due to stress secondary to portal hypertension in the pulmonary endothelium. Other mediators like TNF-α, released by macrophages, activates inducible NO

syntheses (iNOS) increase NO level in the pulmonary circulation(21). There is enough evidence to suggest that NO mediated vasodilatation is important in the HPS pathogenesis. NO is increased in patients with HPS as compared to those without HPS(22). Inhibition of NOS lead to improvement in oxygenation in patients with HPS(23).

In addition CO (carbon monoxide) also plays a role in vasodilatation. CO is primarily produced by heme oxygenase (HO) from degradation of heme. HO is up regulated in pulmonary macrophages, responsible for the production of CO in the pulmonary circulation(24). CO also mediates vasodilatation in the similar way as NO by stimulating cyclic GMP production in arteriolar smooth muscle cells(25).

Diagnossis of HPS:

HPS should be kept in the differential diagnosis of dyspnoeic patients with liver disease. Approximately 50% of patients with HPS complained of dyspnoea. Presence of cirrhosis is also not essential for the diagnosis of HPS. HPS also has been diagnosed in patients with acute alcoholic hepatitis or acute budd chiari syndrome. Platypnoea and/or orthodeoxia may be the more specific symptoms in the diagnosis of HPS. Platypnoea is a type of dyspnoea that increases from the supine to erect position. Orthodeoxia means hypoxia that is worsening on erect position. But sensitivity of all this symptoms is very low(26). Finger clubbing is common in patients with HPS(26). Cyanosis may be noted in

patients with severe HPS as they become sufficiently hypoxic to appear cyanosed at rest. When cyanosis is combined with clubbing in a patient with cirrhosis, finding is highly suggestive of HPS(27).

Diagnosis of HPS required the presence of hypoxia in patients with chronic liver disease with intrapulmonary shunt without evidence of intrinsic cardiorespiratory disorders. Arterial blood gas is used to demonstrate the evidence of hypoxia. It can be repeated after changing the position of patients to demonstrate orthodeoxia.

Determination of hypoxemia:

In the outpatient settings pulse oxymetry can be used to monitor and considered as a screening tool for HPS. The cut off value of ~97% has high sensitivity with moderate specificity in diagnosis hypoxia for an PaO_2 of 70 mmHg. But this method is less sensitive in the diagnosis of mild degree of HPS(28). Arterial blood gas analysis in both sitting and supine position with breathing room air required for confirmation of HPS. The most sensitive marker is the increased alveolar arteriolar oxygen gradient ($PA\text{-}aO_2$). Cut-off values of $PaO_2 < 80$ mmHg or $PA\text{-}aO_2 > 15$ mmHg have been recommended for the diagnosis of HPS. In patients older than 64 years cut off value values of $PaO_2 < 70$ mmHg or $PA\text{-}aO_2 > 20$ mmHg are suggested (29).

Demonstration of intrapulmonary vasodilatation:

Two methods are available to define the intrapulmonary vasodilatation (IPVD).

1) Contrast echocardiography (CE) by microbubble using agitated saline
2) Radioactive lung perfusion scan using macroaggregated albumin (MAA)

Bubble contrast echocardiography is more sensitive and less invasive than the other. It is also easily available as compared to albumin scan. Bubble contrast echo also has advantage of excluding intracardiac shunting(30). It has therefore a choice for screening of HPS. However it must be noted that all those diagnosed to have IPVD by CE may not demonstrate hypoxemia in ABG. In general subclinical HPS patient do not become hypoxemic over time to be considered as HPS(31). In bubble contrast echocardiography, a sample of normally saline is vigorously agitated to produce microbubbles, and then injected into a vein and at the same time tranthoracic echocardiography is being done to look for the appearance of microbubble in the right heart. Normally, these bubbles of size > 25 micrometer in diameter do not cross alveolar capillary bed as they are trapped in the comparatively smaller diameter capillary bed (5–8 micrometer). Appearance of micro bubble in the left heart after intravenous injection suggests that they have been allowed by the dilated pulmonary vessels to reach the left side of the heart. A positive study can of course also occur in patients with cardiac defect like atrial and ventricular septal

defect, but in this case appearance of bubble in the left heart will be much sooner (within three cycles) after their first appearance in the right atrium.

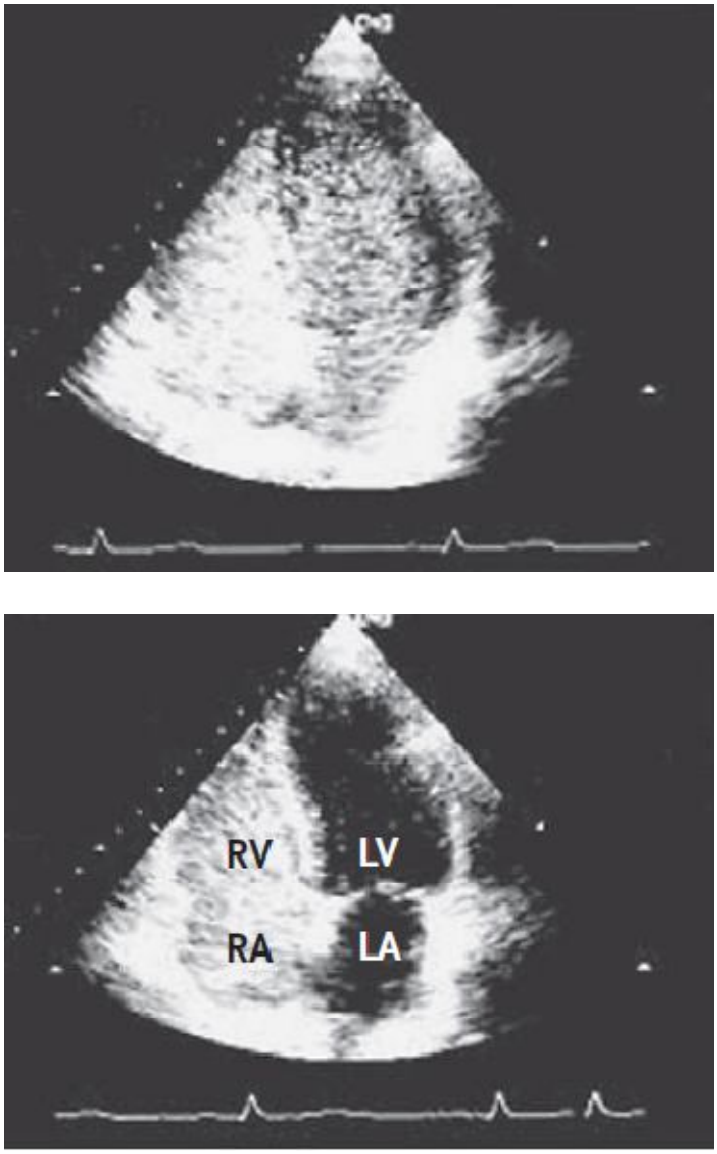


Figure 2: Transthoracic Echocardiographic Features of hepatopulmonary syndrome. (RA: right atrium, LA: left atrium, RV: right ventricle, LV: left ventricle). (The contrast-enhanced echocardiograms show right atrial and right ventricular opacification with microbubbles immediately after injection of microbubble and delayed opacification of the left atrium and left ventricle, respectively.)

MAA perfusion lung scan is done by injection of technetium-99 m radio labelled MAA particles into the peripheral vein, followed by scanning of the whole body to estimate the extra pulmonary shunt fraction. These radio-labelled particles of diameter of 10–90 mm and are trapped in the normal pulmonary circulation. Thus, the detection of a significant amount of radiation in the brain or kidneys suggests intrapulmonary vasodilatation or intracardiac shunting. One drawback of this study is unable to differentiate between extra cardiac and intracardiac shunting. It is a highly specific but less sensitive than bubble contrast echo for detecting IPVD consistent with HPS and in the absence of hypoxemia it usually unable to detect intrapulmonary vasodilatation (30). At the same time it can quantify the amount of intrapulmonary vasodilatation in contrast to CE which has the advantage of being high sensitivity(30).

Diagnosis of hepatopulmonary syndrome:

1) Liver disease
2) Alveolar-arterial oxygen gradient >15 mmHg
3) Demonstration of intrapulmonary shunting

Prognosis:

Cirrhotic patients with HPS have higher mortality as compared to the patients without HPS with the same severity of liver dysfunctions (32)(27). Five years

survivability of patients with HPS not underwent liver transplantation is only 23% compared to patients without HPS(33). Survivability also depends on the severity of HPS. Severe HPS that is $PaO_2 < 60$ usually have very bad prognosis and most of them died within 6 months(32). That's why extra MELD points have been allocated to these groups of patients while listing for liver transplant. All these patients may not die respiratory failure but it has been noted that most of them died due to liver failure and its complications.

Treatment:

Liver transplantation is the only available effective therapy for the HPS. Several pharmacologic therapies have been tried in different uncontrolled trial with unsuccessful result(34).

Medical therapies:

1) Garlic preparation(35)
2) Nitric oxide inhibitors(36)
3) Pentoxifyllene(37)
4) Cycloxygenase inhibitors
5) Systemic glucocorticoids
6) Cyclophosphamide

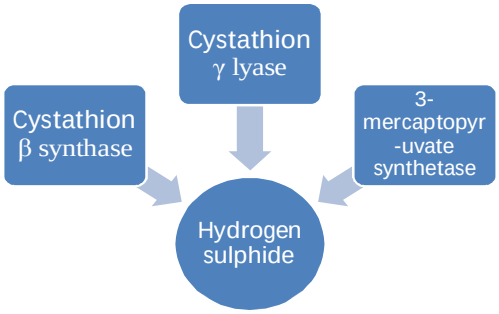
Liver transplantation is the only effective treatment options available for HPS. Survival after liver transplantation is similar to the patients undergoing

transplantation without HPS except for those with severe hypoxemia (PaO_2 <60%). Patients with severe hypoxemia noted to have increase peri-transplant and post transplant mortality and also the increase interval between resolution of hypoxemia and transplantation. Long term survivability is not much different from the patients without HPS after liver transplantation(12). Hypoxemia is the only strongest predictor of mortality after liver transplantation(38). Because of poor outcome without liver transplantation diagnosis of hepatopulmonary syndrome with less than 60 mm of Hg has been given a priority for liver transplantation(39). Recently published data suggests improving outcome after liver transplantation in patients with HPS due to better postoperative care particularly in the immediate post transplant period. Mortality of only 9% has been reported in a recently published trial included patients with severe HPS (PaO_2 < 50 mmHg).

Hydrogen sulphide:

Hydrogen sulphide (H_2S) is a colourless, water soluble gas with unpleasant smell of rotten egg(40). It is a small molecule and can permeate membranes freely (41). Endogeneous H_2S is synthesized in various mammalian tissues from L cysteine by two enzymes, cystathion-β-synthetase (CBS) and cystathionine-γ-lyase (CSE), with the help of the co-factor pyridoxal-5-phosphate(42) . It was initially reported that H_2S is a toxic gas and an environmental pollutant (40) with minor physiological or pathological significance. But, in recent years, H_2S has gained importance as a physiological gaseous mediator, which is involved in many cell signaling processes(43) . Evidence indicates that H_2S plays important roles in many pathological conditions such as hypertension, inflammation, edema, gastric mucosal ulceration and hemorrhagic shock(42). Inflammation is mediated by increased leukocyte adhesion to the injured site and H_2S donors can suppress leukocyte adherence to the vascular endothelium and reduce leukocyte infiltration(44). H_2S is mainly metabolized to sulfate and thiosulfate via oxidation in the mitochondria. Rhodanese is a mitochondrial enzyme involved in the detoxification of H_2S (45). Studies have shown that the administration of physiological level of H_2S attenuates the myocardial injury and protects blood vessels and l

imits inflammation(46).



Enzymes involved in H_2S biosynthesis

Figure 3: Enzymes involved in H_2S biosynthesis

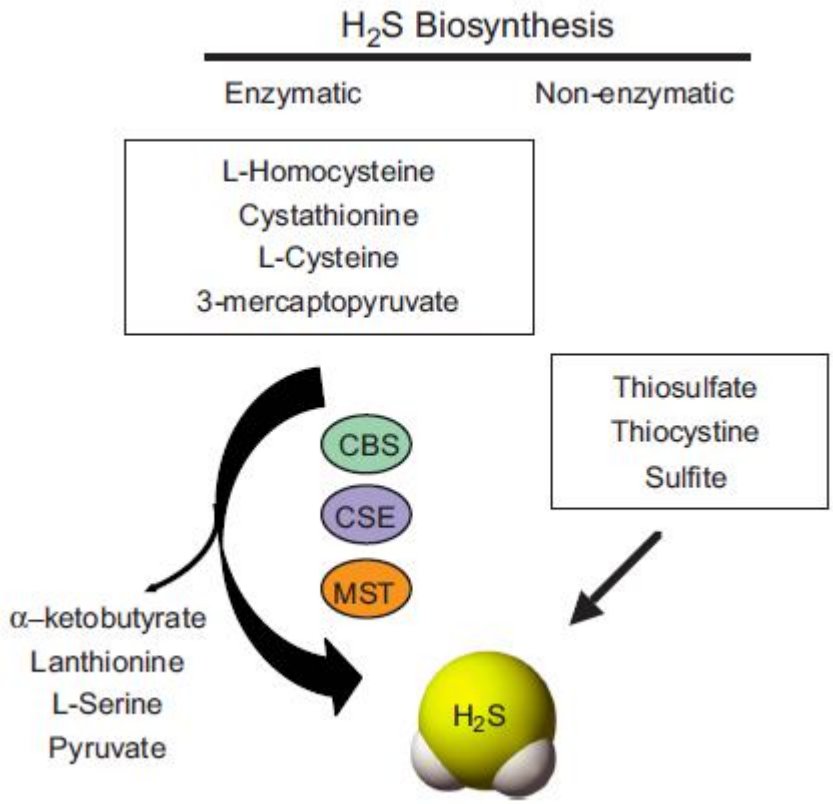


Figure 4: Enzymatic and non enzymatic pathways of hydrogen sulphide (H_2S) production (CBS: cystathion-β-synthetase, CSE: cystathionine-γ- lyase, MST: 3 mercapto pyruvate synthetase)

Various studies have shown protective effect of H_2S in colonic mucosal healing in inflammatory bowel disease(47).

Role of H_2S in portal hypertension has been studied in the recent past by Fiorucci et al and decrease level of tissue level was found in the patients with portal hypertension and also there has been decrease in the expression of cystathion gamma lyase at the tissue level in patients with portal hypertension(41). The biological mechanism and pathways of sulphide Norris et al has shown the effect of H_2S on the hepatic microcirculation and contribution of H_2S in microcirculatory dysfunction during sepsis(48). Fiorucci et al first investigated the H_2S role in patients with portal hypertension(41). They demonstrated that expression of CSE in hepatocytes and hepatic stellate cells (HSCs) also known as Ito cells, but importantly not in the sinusoidal endothelial cells (SECs) is important in the regulation of portal pressure. Norepinephrine infusion in a K_{ATP} channel dependent increase in hepatic resistance has been antagonised by hydrogen sulphide by infusing NAHS. It was concluded that endogenous H_2S would function as a vasodilator, preserving blood flow. Work by Norris et al.(49) Mentioned that H_2S also have differential

effects on different sites of the liver like sinusoidal and presinusoidal levels. As liver contains both venous and arteriolar blood supply, regulation of hepatic microvasculature is complex. Intricate interplay of multiple vasoregulators along with hepatic sinusoidal cells, sinusoidal endothelial cells and kupffer cells contribute to hepatic blood flow regulation. Normal sinusoidal pressure is being maintained by combined effect of endothelin-1, nitric oxide and carbon monoxide. Endothelial ET-1 binds to the ETA receptor, which is primarily expressed by HSCs(50). Sinusoids are surrounded by these cells and contraction of these cells lead to reduction in sinusoidal blood flow. Endothelial nitric oxide synthase activation and subsequent relaxation of sinusoids occurred by endothelial nitric oxide synthase (eNOS) after binding to endothelin B1 receptors which were expressed by sinusoidal endothelial cells. Constitutively expressed parenchymal heme oxygenase-2 (HO-2) is responsible for the maintatinance of basal carbon monoxide level. Increase in intracellular cyclic guanosisne monophosphate after intraction with guanylyl cyclase is done by both NO and CO. NO has been shown to limit the release of ET-1 and to increase the expression of endothelial HO-1 (51).

Signalling pathways involved in the pro-angiogenic effect of H_2S:

Activation of multiple signalling pathways to initiate angiogenesis

1) PI-3K/Akt axis(52)
2) MAPK pathway (extracellular signal related kinase ERK ½ and p38(53)

3) Modulation of hypoxia inducible factor 1α (HIF-1α)(54)

4) Up-regulation of VEGF expression(53)

5) Opening of K_{ATP} channels(55)

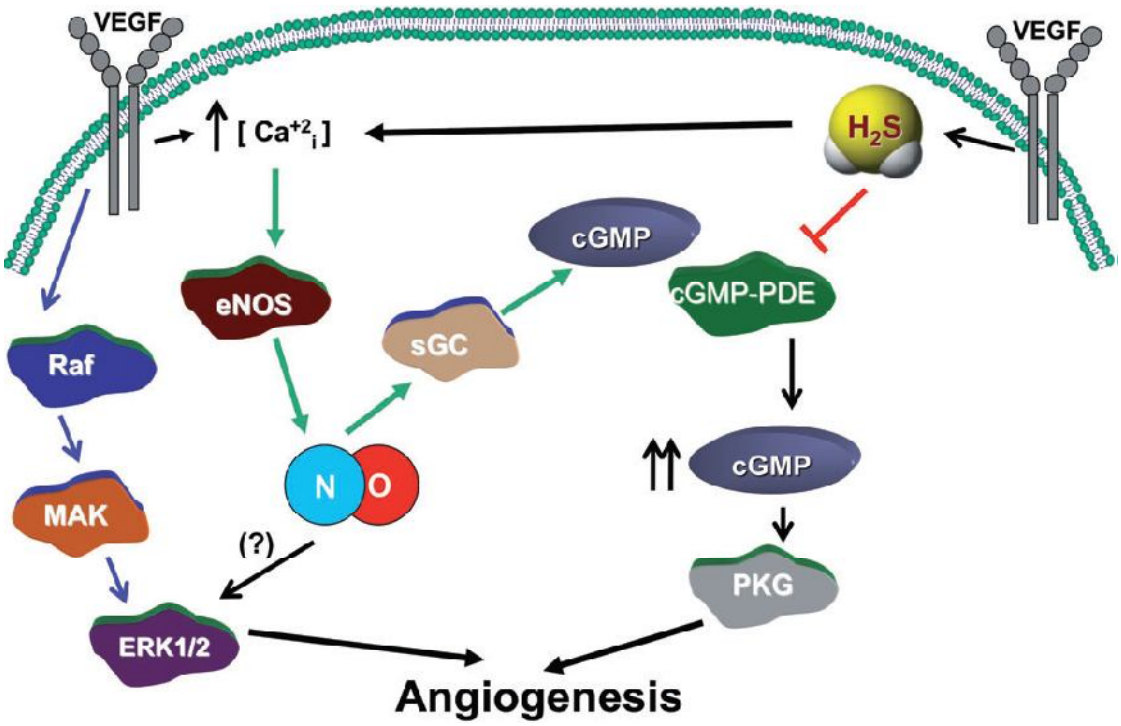


Figure 5: Pathway of angiogenesis

Yang et al demonstrated that VEGF signalling which is dependent on H_2S, act by increasing intracellular calcium level and subsequent increase in CSE activity by calcium calmodulin pathway(56). Whatever may be the mechanism role of H_2S has been established in the vasodilatory, anti-inflammatory and angiogenic activity.

AIM

Aim 1: To study the prevalence of hepatopulmonary syndrome in the patients with cryptogenic chronic liver disease.

Aim 2: To elucidate association of plasma hydrogen sulphide with hepatopulmonary syndrome in patients with cryptogenic chronic liver disease.

METHODOLOGY

Study Design: Observational study

Study Period: March 2012 to January 2014

Study Setting: Department of Hepatology

Christian Medical College

Study patients:

All patients with cryptogenic chronic liver disease were enrolled prospectively in this study. Cryptogenic chronic liver disease was diagnosed as per following criteria-

1) Presence of chronic liver disease with or without portal vein thrombosis as evidenced by clinical, biochemical, radiologic and endoscopic findings
2) Etiological evaluations for viral (HBsAg and anti HCV antibody), autoimmune (Serological profiles like ANA, ASMA, Anti LKM, AMA and SLA), wilson's disease (Serum ceruloplasmin, 24 hour urinary copper and KF ring), haemochromatosis (ferritin and percentage of

transferring saturation) and non alcoholic fatty liver disease (bdy mass index and metabolic syndrome) have been excluded.

Exclusion criteria:

1) Significant pulmonary abnormality e.g. massive pleural effusion and obstructive airway disease.
2) Presence of intracardiac shunt like atrial and ventricular septal defect.
3) Inability to get informed consent

Consent: Informed consent was taken from all the patients

Study protocol was approved by Institutional review board (IRB)

Methods:

Demographic data:

Demographic data like age, gender and residence were collected.

Baseline investigations:

The following base line investigations were done in all study subjects

1) Etiological workup for chronic liver disease (Viral serology for hepatitis B and C, if negative autoimmune profile that included ANA,SLA,LKM,SMA, Wilson's workup that included 24 hours urine copper and serum ceruloplasmin, Iron studies)
2) Ultrasonography of abdomen and Doppler of hepatic vein, portal vein, splenic vein and inferior vena cava.
3) Gastroscopy to assess varices.
4) Ascitic fluid analysis if ascites was present.
5) Liver biopsy either percutaneous or transjugular route as indicated on a case to case basis.
6) Baseline biochemical investigations like liver function tests and renal function test, alpha fetoprotein, prothombin time with INR and complete blood counts.

Evaluation for HPS in study patients:

All study patients underwent arterial blood gas (ABG) analysis and contrast echocardiography (CE) to look for the evidence of hypoxemia and intrapulmonary vasodilatation (IPVD) respectively.

Arterial blood gas analysis for the determination of alveolar and arteriolar oxygen gradient P (A-a) O_2:

Arterial blood gas sample was obtained by percutaneous radial artery puncture with the subject in a standing position (at least for 5 minutes) breathing room air and was analysed with a standard blood gas analyser (BG Electrolytes, Instrumentation Lab. Inc., USA). Determination of difference between alveolar oxygen pressure (P_AO_2) and arterial oxygen pressure (P_aO_2) was done using following formula:

$$P_AO_2\text{-}P_aO_2 = (F_IO_2\,[P_{atm}–PH_2O] – [P_aCO_2/0.8]) – P_aO_2,$$

- P_AO2: partial pressure of alveolar oxygen
- P_aO_2: partial pressure of arterial oxygen
- F_IO_2: fraction of inspired oxygen
- P_{atm}: atmospheric pressure
- PH_2O: partial pressure of water vapour at body temperature
- P_aCO_2: partial pressure of arterial carbon dioxide

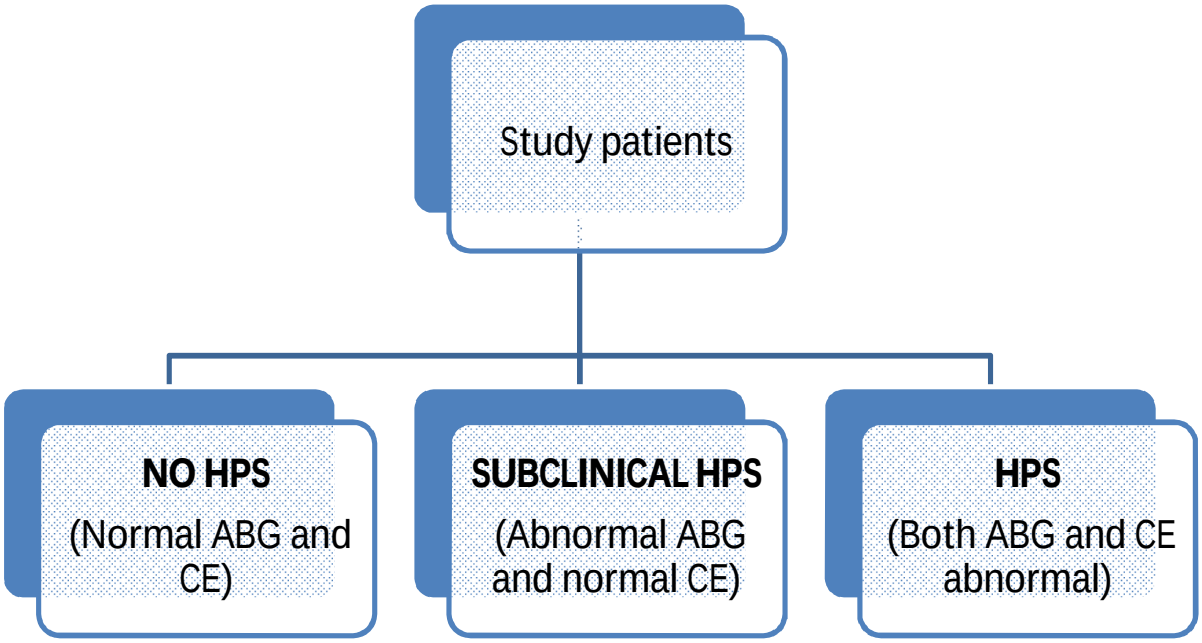
(0.8 corresponds to the standard gas-exchange respiratory ratio at rest); the normal range is 4 to 8 mm Hg (0.5 to 1.1 kPa)

The normal range for the partial pressure of oxygen is 80 to 100 mm Hg (10.7 to 13.3 kPa) at sea level, while the patient is at rest and breathing ambient air.

Contrast echocardiography (CE):

Contrast echocardiography (CE) was performed by using a General Electric echocardiography device with 3- and 5-MHz probes with patients lying in a supine position. Intravenous access was done in right cubital vein. Agitated saline was used to produce microbubble of >10 micrometer diameter and was injected in the right arm. Appearance of microbubble within three to six cycles after appearance of the same in right heart signifies intrapulmonary vasodilatation (IPVD). Normally diameter of pulmonary capillaries are less enough to restrict these microbubble to pass through the capillaries which is less than 8 to 15 micrometer.

Study patients were classified into three groups on the basis of ABG and CE findings as mentioned below.



	NO HPS	SUBCLINICAL HPS	HPS
P (A-a) O_2	Normal	Normal	Abnormal
Contrast Echocardiography	Normal	Abnormal	Abnormal

Study patients were also grouped as No IPVD (negative contrast echocardiography) and IPVD (positive contrast echocardiography). Patients

with IPVD that is positive contrast echocardiography were divided into mild and moderate to severe according to the filling of the right heart three cycles after appearance of contrast in the left heart. It was considered as mild when less than 25% of the right heart was filled by contrast agent and moderate to severe if it is more than 25%.

everity of HPS was defined by the following criteria:

Degree of severity	Alveolar-arterial oxygen gradient [P (A-a) O_2]	partial pressure of oxygen (P_aO_2)
Mild	≥15 mm Hg	≥80 mm Hg
Moderate	≥15 mm Hg	≥60 to <80 mm Hg
Severe	≥15 mm Hg	≥50 to <60 mm Hg
Very severe	≥15 mm Hg	<50 mm Hg

Adapted from reference no : N Engl J Med 2008; 358:2378-87.

Measurement of Plasma H2S Level:

Blood sample (5ml) was collected from the subjects and H2S levels in plasma was measured spectrophotometrically ten minutes of collection of blood sample according to following method. 100µl of aliquots of the samples was mixed with 50µl of distilled water in micro-centrifuge tubes containing 300µl of zinc acetate (1% w/v) to trap H2S. The reaction was terminated after 5 min by adding 200µl of N, N-2dimethyl-p-phenylenediamine sulphate (20mM in 7.2 M HCl) and immediately followed by addition of 200µl of FeCl3 (30mM in 1.2 M HCl). The mixture was kept in the dark for 20 minutes. In order to precipitate protein from the samples 150µl of trichloroacetic acid (10% w/v) was added. The mixture was then centrifuged at 10,000 rpm for 10 minutes. The absorbance of the resulting supernatant was measured at 670 nm using a 96-well plate reader (Bio-Tek instruments, INC, USA). Finally, H2S concentration in the plasma was calculated against the calibration curve of standard H2S solutions (NaHS: 3.125-100 µM).

Potential bias:

Consecutive patients were included in the study to exclude selection bias. Another potential source of bias would be the difference in aetiology of chronic liver disease, so only patients with cryptogenic liver disease with or without portal vein thrombosis were included.

Statistical analysis:

Sample size: Study design was an observational study. With an assumption that 20% would be the prevalence of hepatopulmonary syndrome in patients with cryptogenic CLD patients sample size of approximately 20 patients in each group with total of 60 patients with precision of 10% and desired confidence of 95%.

Statistical Methods:

Data were analysed using three groups:

NO HPS: Normal P_AO_2- P_aO_2 and normal CE

SUBCLINICAL HPS: Normal P_AO_2- P_aO_2 and abnormal CE

HPS: Abnormal P_AO_2- P_aO_2 abnormal CE

Continuous data was expressed as median and range and categorical data was expressed as number and percentage. The difference in H_2S values were assessed by Mann-whitney U and Kruskal Wallis test in different groups.

Plasma H2S level in patients with IPVD was compared to patients with no IPVD after adjusting for MELD score (binary logistic regression).

Spearman correlation (ρ) was used to assess the correlation between MELD, PaO2and H2S.

For all comparisons, statistical significance was defined as a P value less than 0.05.

Funding:

Fluid research grant, Christian Medical College.

RESULTS

Prevalence of HPS in study patients:

Total no of study patients: 58

Number of patients without HPS (NO HPS): 24

Number of patients with subclinical HPS (SUBCLINICAL HPS): 21

Number of patients with HPS (HPS): 13

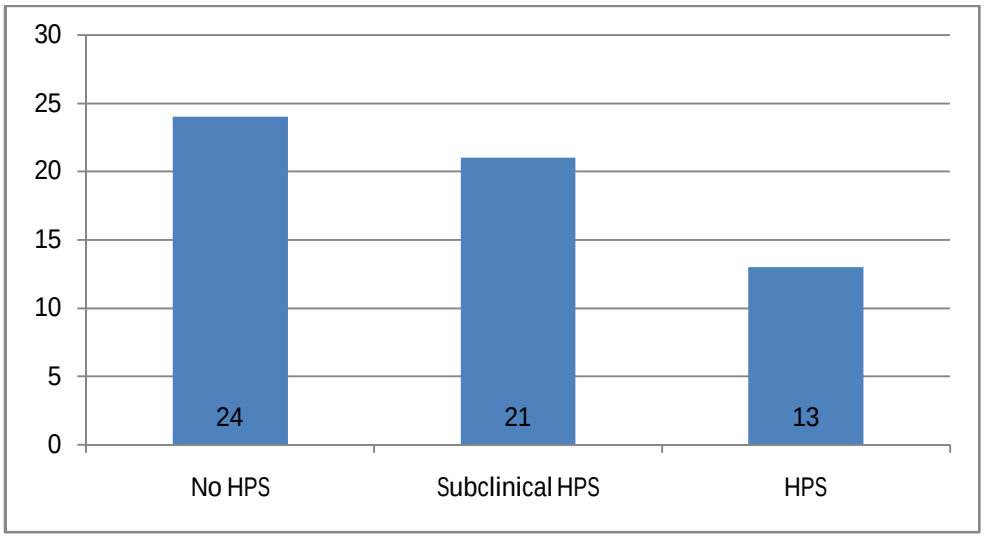


Figure 3: Distribution of patients in three groups

Severity of HPS in study patients:

Severity of HPS among study patients according to PaO2 were mentioned in figure 2. Severity was defined by the following criteria:

1) Mild – $PaO_2 \geq 80\%$
2) Moderate - $PaO_2 \geq 60\%$ - <80%
3) Severe - $PaO_2 \geq 50\%$ - <60%
4) Very severe - <50%

Five patients had moderate and four had mild HPS. Severe and very severe HPS was noted in two patients each.

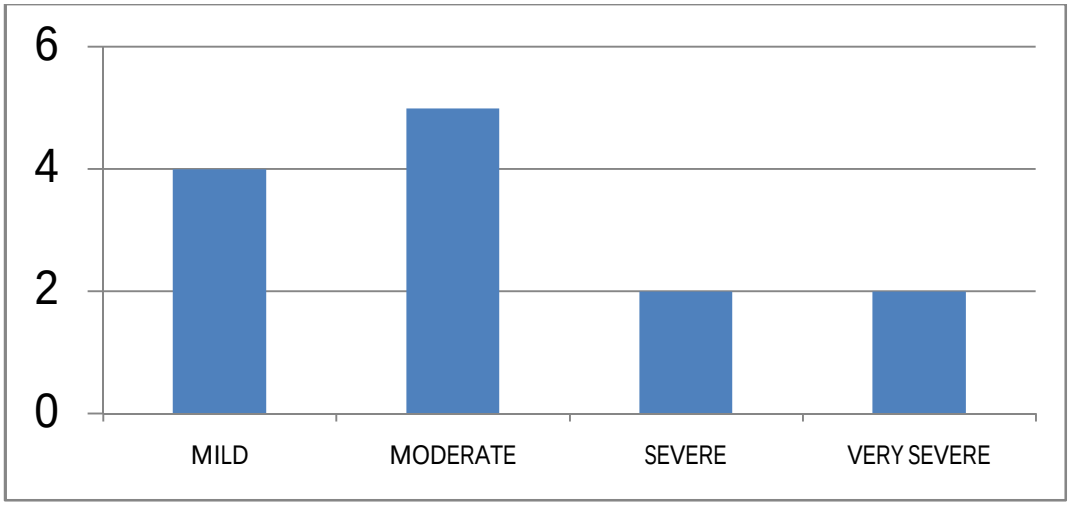


Figure 4: Severity of HPS according to PaO2 (n=13)

Demographic data of the study patients :

Median age of 58 study patients was 45 years (range 16-74 years). Median age of the patients without HPS, with subclinical HPS and with HPS was 46, 46 and 40 years respectively. There were predominantly male in all three groups.

Table 1: Demographics in study patients (n=58) with cryptogenic chronic liver disease:

	Total (n=58)	No HPS (n=24)	Subclinical HPS (n=21)	HPS (n=13)
Age in years Median (Range)	45 (16-74)	46 (25-69)	46(20-74)	40 (16-68)
Gender (M:F)	45:13	16:8	17:4	12:1

Clinical parameters:

Clinical features associated with the patients with HPS are shown in table ... Dyspnoea was noticed in only two patients (one in the subclinical and other in the HPS group). Cyanosis was observed in one patient with HPS. Only one patient in the HPS group had spider naevi. Clubbing was noted in 5 patients (2 in the subclinical and 3 in the HPS group).

Table 2: Clinical parameters in the study subjects (n=58)

	Study patients (n=58)	No HPS (n=24)	Subclinical HPS (n=21)	HPS (n=13)
Cyanosis	1	0	0	1
Clubbing	5	0	2	3
Spider naevi	1	0	0	1
Dyspnoea	2	0	1	1

Baseline laboratory parameters:

Consecutive patients with cryptogenic chronic liver disease were included in the study. Patients with HPS had higher prothombin time and lower albumin level as compared to other groups. There was thrombocytopenia in all the three

groups with lowest in the group with HPS. Haemoglobin and aminotransferase level was similar in all three groups.

Table 3: Baseline laboratory parameters in the study patients (n=58)

	Study patients (n=58)	No HPS (n=24)	Subclinical HPS (n=21)	HPS (n=13)
Haemoglobin (gm/dl)	11 (6.1-15.5)	11.2 (6.7-14.8)	10.2 (6.1-15.5)	12 (7.8-15.5)
Albumin (gm/dl)	3.4 (1.3-4.8)	3.5 (2.3-4.8)	3.1 (1.3-4.1)	2.9 (2.2-4.6)
Platelet count x 10^3 / cu. mm	78 (18-268)	95 (36-268)	72 (18-193)	55 (22-106)
Serum bilirubin (mg/dl)	2.3 (0.3-17.8)	1.7 (0.3-4.9)	2.6 (0.6-17.8)	3.1 (0.7-5.5)
AST (u/L)	49 (16-102)	44 (16-88)	53 (32-102)	51 (20-75)
ALT (u/L)	29 (2-117)	30 (2-117)	30 (11-85)	25 (8-54)
PT (seconds)	14.1 (11-24)	12.9 (11.1-17.7)	14.5 (11-19.2)	15.7(12.8-24)

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, PT: Prothombin time

Liver disease severity and oxygenation in study patients:

Most of the patients in all three groups were in Child's class A. Overall Child's class A, B and C in all the three groups were 30, 18 and 10 respectively. There was more number of patients with child's class C in the subclinical group of patients as compared to others. The median MELD score in three groups were 11, 13 and 14 respectively. In patients with HPS median MELD score was 14 with a range of 10 to 20 which was higher than other two groups. Low PaO_2 was noted in the patients with HPS as compared to others.

Table 4: Child's class, MELD and PaO_2 in study patients:

	Study patients (n=58)	No HPS (n=24)	Subclinical HPS (n=21)	HPS (n=13)
Child's class (A/B/C)	30/18/10	14/9/1	9/5/7	7/4/2
MELD score Median (range)	12 (7-27)	11 (7-16)	13 (7-27)	14 (10-20)
PaO_2	92 (42-138)	93.8 (42-138)	98.5 (83-127)	76 (42-133)

MELD: Model for End Stage Liver Disease, PaO_2: Partial pressure of oxygen

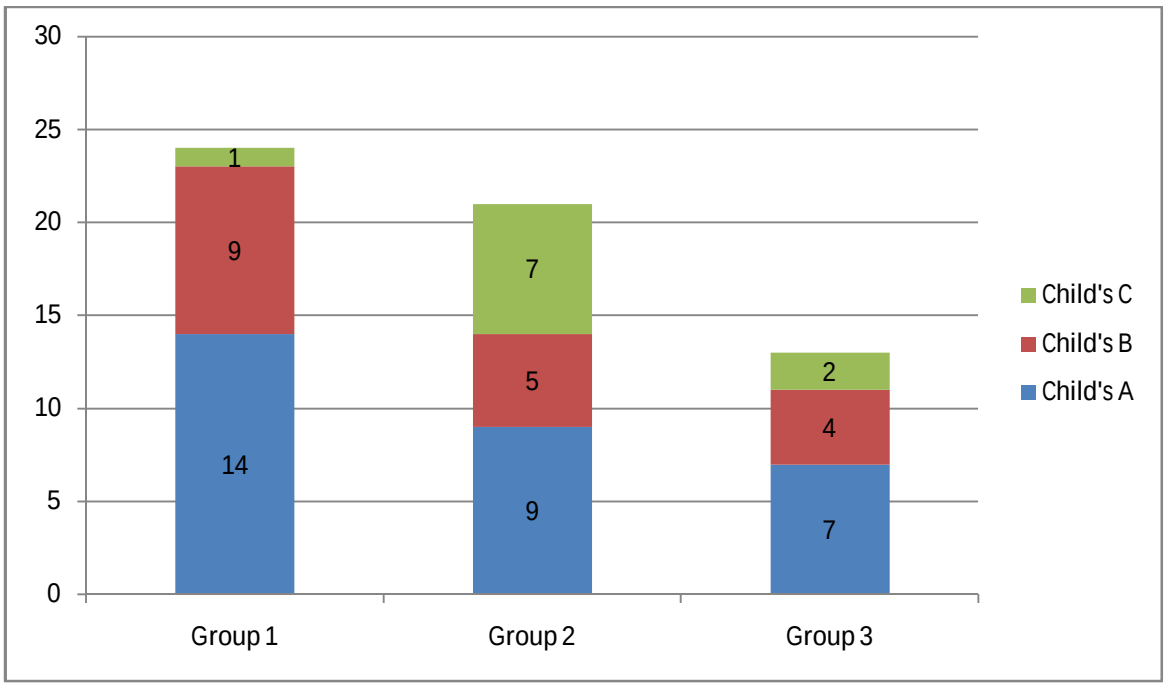


Figure 5: Child's class status in three groups

Prevalence of IPVDs, Hypoxia and hepatopulmonary syndrome:

Intrapulmonary vasodilatation (IPVD), as defined by microbubble opacification of the left atrium within three to six cardiac cycles after right-atrial opacification, was noted in 34 patients by contrast echocardiography. 13 patients had hypoxemia (PaO_2 <80%) on ABG. All patients with hypoxemia had demonstrable IPVD on contrast echocardiography. HPS was diagnosed in these thirteen patients. Chest X ray was normal in all of the patients.

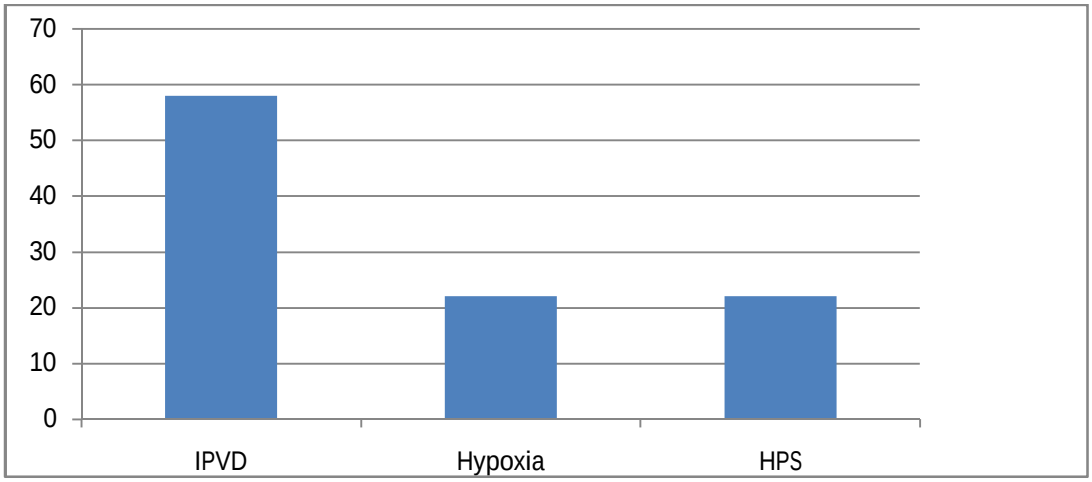


Figure 6: Percentage of patients with IPVD, hypoxia and HPS among study patients (n=58)

Plasma hydrogen sulphide level in the different groups:

Plasma hydrogen sulphide level was 12.3, 19.6 and 19.5 in patients without HPS, with subclinical HPS and with HPS respectively. As compared to patients with no HPS, patients with subclinical HPS had higher H2S levels (p-value: 0.03) and there was a trend to higher H2S in patients with HPS (p-value: 0.3). Plasma level of H_2S level was similar in patients with subclinical HPS and HPS.

Table 5: Plasma H2S in different groups (n=47)

	Study patients (n=47)	No HPS (n=19)	Subclinical HPS (n=20)	HPS (n=8)	P value
Plasma H_2S (µmol/L) Median (range)	16.4 (0-83)	12.3 (0-47)	19.6 (5.7-83)	19.5 (6.4-64.3)	0.09

HPS : Hepatopulmonary syndrome

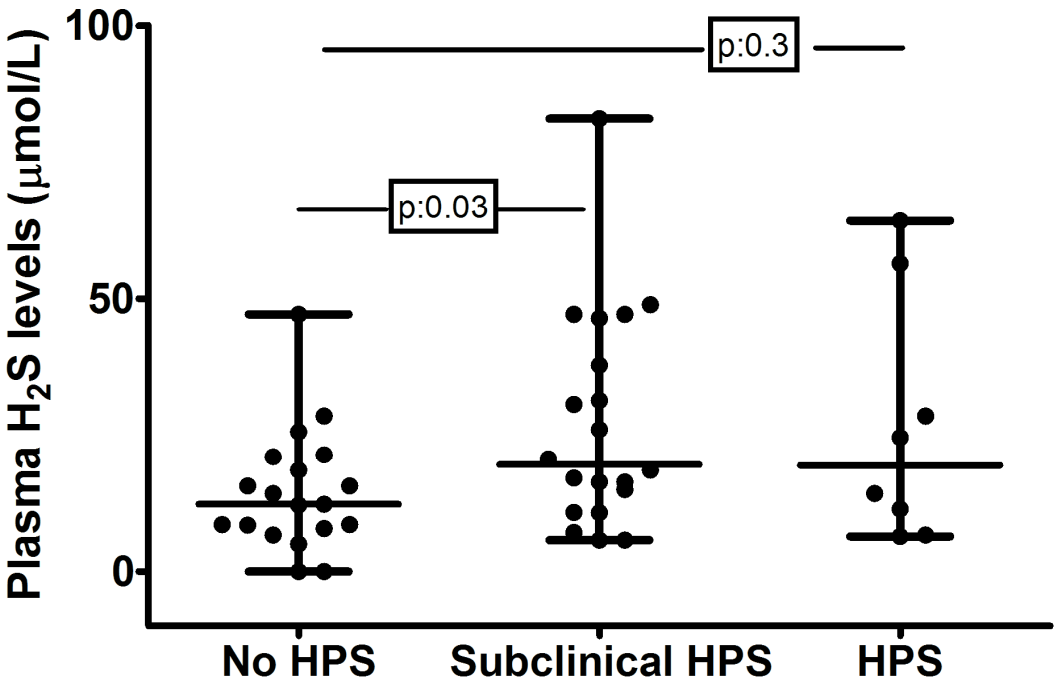


Figure 7: Plsma H_2S in different study patients (n=47)

Plasma H_2S, PaO_2 and MELD score in patients with and without IPVD:

According to contrast echocardiography finding study patients were divided into two groups i.e. with IPVD and without IPVD. Plasma H_2S level was significantly higher in the patients with IPVD (p=0.03). After adjusting for MELD score, there was trend, albeit not reaching statistical significance, towards higher H2S levels in patients with IPVD as compared to patients with no IPVD (p-value : 0.07)

Table 6: Plasma H2S, PaO_2 and MELD in patients with IPVD and without IPVD

	With IPVD (n-34)	Without IPVD (n=24)
MELD Median (Range)	13 (7-27)	10 (7-16)
pAO_2 (mm of Hg) Median (Range)	91.5 (42-133)	93.8 (42-138)
H_2S (µmol/L) Median (Range)	19.6 (5.7-83)	12.3 (0-47)

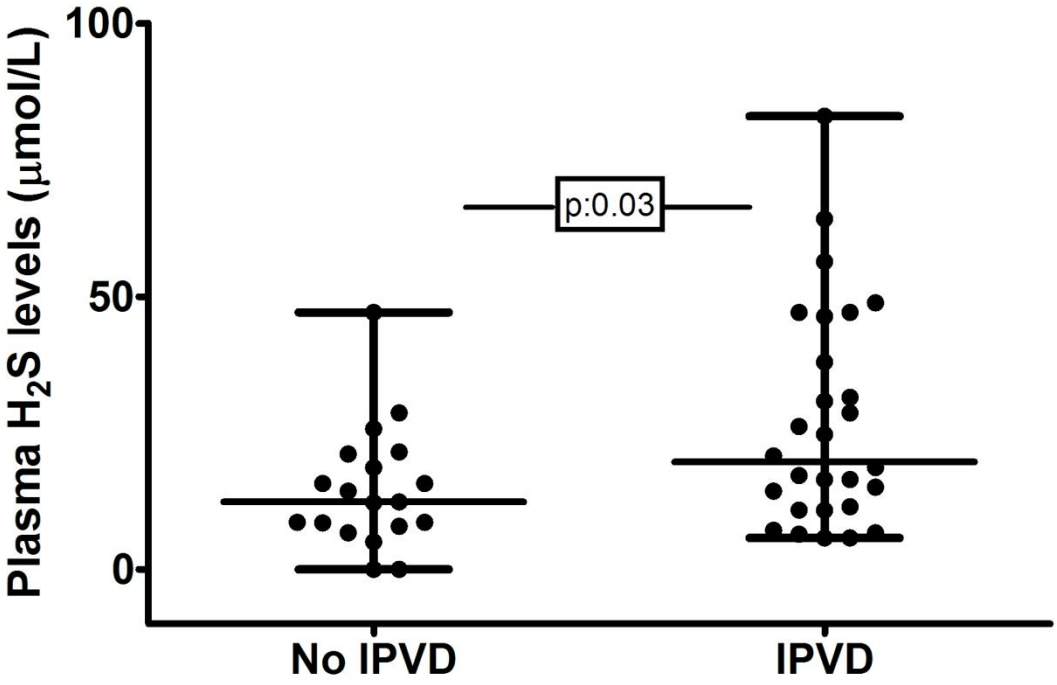


Figure 8: Plasma H_2S, PaO_2 and MELD in patients with IPVD and without IPVD

Plasma H_2S level in the different grades of IPVDs:

Subjective assessment of the appearance of contrast in the left heart after injection of contrast in the right cubital vein was done. It was considered mild when less than 25% of the right heart was filled with contrast and more that that was considered moderated to severe. Increase in the severity of IPVD was associated with higher level of H2S level (13.2 Vs 26.1) although this difference was not statistically different.

Table 7: Plasma H_2S level in the different grades of IPVDs:

	Mild (<25%) (n=10)	Moderate to severe (>25%) (n=17)	P value
H_2S	13.2 (5.7-47)	26.1 (5.7-64.3)	0.07

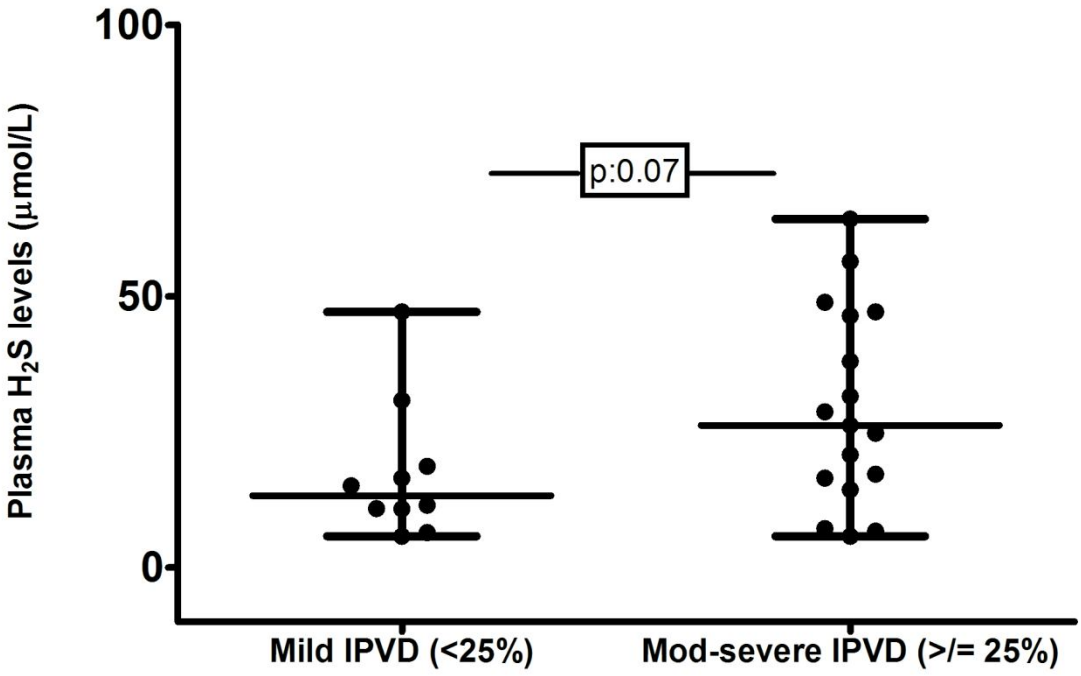


Figure 9: Plasma H2S level in the different grades of IPVDs

Correlation between Plasma H_2S level, PaO_2 and MELD

On bivariate correlation analysis, there was a moderate positive correlation between MELD score H_2S levels (ρ = 0.47; p-value: 0.001). There was no correlation between H2S (ρ: -0.13-; p-value: 0.39) and MELD score (ρ:-0.94; p-value: 0.49) to PaO_2.

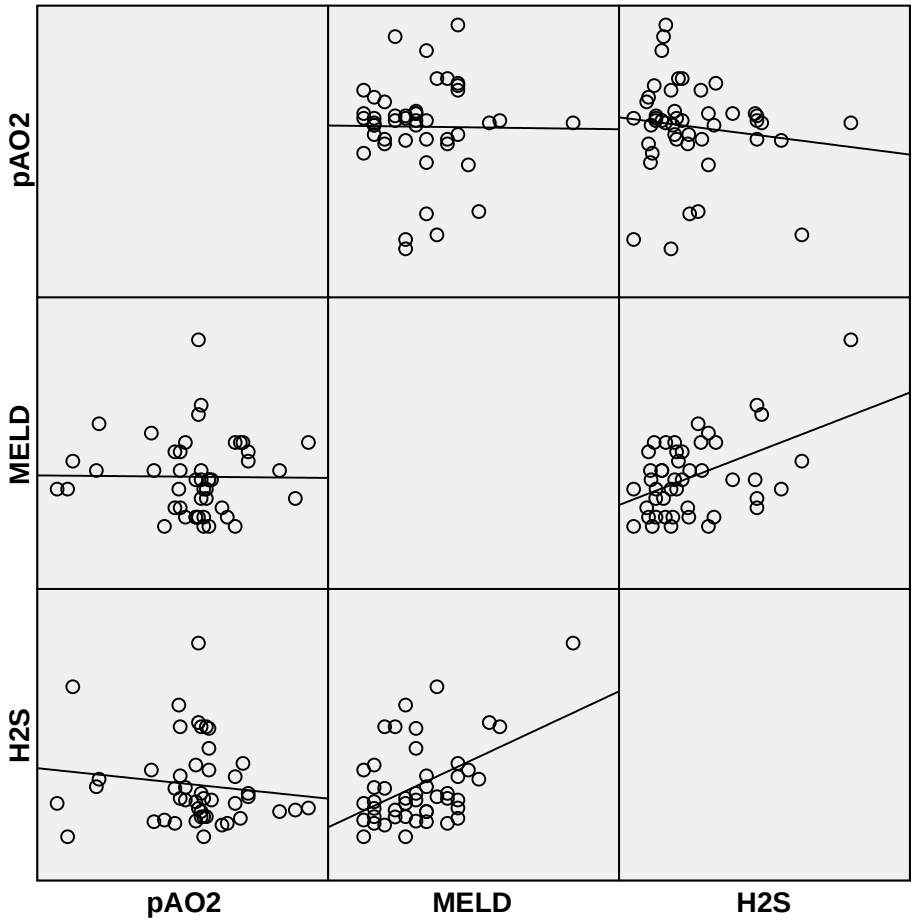


Figure 10: Correlation between plasma H_2S, PaO_2 and MELD score

DISCUSSION

In this study we enrolled consecutive cryptogenic chronic liver disease patients with or without portal vein thrombosis to study the prevalence of HPS. We found the frequency of subclinical HPS and HPS was 36.2% and 22.4% respectively. IPVD was detected in 59% of the study patients.

We also noted a higher plasma H_2S levels in patients with subclinical HPS as compared to patients with no HPS. In patients with HPS, H_2S levels were similar to patients with subclinical HPS. This may be secondary to less number of patients with HPS in the study group. Significantly higher plasma H_2S level was also noted in patients with IPVD as compared to patients without IPVD. This difference in the plasma H_2S level was not significant after adjusting MELD score may be due to less number of patients in the HPS group. Positive correlation noted between MELD score and plasma H_2S levels needs further larger studies to corroborate.

There are very few studies regarding the prevalence of HPS in patients with chronic liver disease. It has been estimated to be around 10 to 30% patients undergoing evaluation for liver transplantation. As patients are not evaluated for the HPS unless they are symptomatic in the form of dyspnoea, cyanosis or clubbing; we tend to underestimate the true prevalence. In our prospective study, even in predominantly Child's class A patients the prevalence of HPS was 22%. In addition, 59% patients had demonstrable IPVD. The prognosis,

quality of life and further natural history in these patients needs to be elucidated in a follow up cohort study.

Screening for HPS is done by contrast echocardiography for IPVD and ABG to look for the evidence of hypoxemia. Another modality to detect the presence of IPVD is 99m technetium aggregated albumin scan which is less sensitive as compared to contrast echocardiography in detecting IPVD(1). We used contrast echocardiography in our study. Semi quantitative assessment of contrast density was done subjectively to determine the grade of IPVD(2).

Subclinical HPS is the form of HPS where patient has a detectable IPVD without hypoxia. These patients may need frequent monitoring for the development of hypoxemia and overt HPS. The strategy of follow up is not well characterised and needs further research.

Although clinical outcomes are similar in patients with or without IPVD after liver transplantation(3). Mimidis et al studied in 75 patients with CLD with Child class A and B for the evidence of IPVD by CE. Eight of 75 (10.7%) patients had a positive CE without evidence of hypoxemia(4). In a study by Shafiq et al frequency of subclinical and overt HPS were 13.2% and 15.8% respectively(5). In the same study all of patients with HPS were CTP class C and HCV related cirrhosis. Prevalence also varies depending on the cut off for arterial oxygenation used for the diagnosis of HPS. In a study by Schenk et al the prevalence of HPS was considerably higher using alveolar arteriolar oxygen

difference as an indication of hypoxemia. The prevalence was considerably higher using $P(A\text{-}a)O_2$ (>15 mm Hg, 32%; >20 mm Hg, 31%; and >age related threshold, 28%) than using reduced partial pressure of arterial oxygen PaO_2 as a threshold (<80 mm Hg, 19%; <70 mm Hg, 15%; and <age related threshold, 15%)(6). Prevalence of HPS was also noted to be higher in patients with CLD as compared to patients with extra hepatic portal venous obstruction (EHPVO) (18.5% Vs 4%)(7). In our study prevalence of subclinical HPS and HPS was noted to be higher than the previously reported. IPVD was noted to be more common in patients with alcoholic cirrhosis and with advanced liver dysfunction(1). Similarly frequency of HPS is much higher in cirrhosis than in extrahepatic portal venous obstruction(7). In the same study by Gupta et al child class and portal vein diameter were the only two risk factors significantly associated with the development of IPVD.

Role of various vasodilatory gaseous transmitters like NO and CO have been evaluated in the pathogenesis of HPS. Although role of H_2S was studied in the setting of portal hypertension its role in HPS has not been studied. H_2S is considered to be a vasodilator and also plays a role in angiogenesis(8). It has been shown that CSE derived H_2S is involved in the maintenance of portal venous pressure. The reduction of CSE expression in the liver with cirrhosis contributes to the development of increased intrahepatic resistance and portal

hypertension(9). Precapillary and capillary pulmonary vasodilation and angiogenesis are the two important mechanism in the development of IPVD and subsequent HPS.

Significant difference in the level of plasma H_2S was noted in patients with and without IPVD ($p=0.03$) suggest possible role of H_2S as a vasodilator in IPVD. H_2S level was also much higher in patients with moderate to severe IPVD as compared to patients with mild IPVD.

Mucosa of the small and large intestine produces H_2S In the small and large intestine(10). Many of the bacteria within the lumen can produce H_2S in close proximity to the epithelium(11). The colonic mucosa can detoxify H_2S that it is exposed to(12). In this way intestinal epithelium can produce H_2S along with it may serve as metabolic barrier to the diffusion of bacteria derived H_2S into the lamina propria(13). Within the epithelium, H_2S is rapidly inactivated by a complex of mitochondrial enzymes collectively referred to as the “sulfide oxidation unit”(14). Colonocytes can adapt itself to utilyze H2S as an energy source and at the same time it has the ability to detoxify the same(15)(16)(17).

CONCLUSIONS

- In 59% patients of cryptogenic cirrhosis there was a demonstrable IPVD.
- The prevalence of HPS in patients with cryptogenic cirrhosis was 22%.
- Plasma H2S levels were higher in patients with IPVD and HPS as compared to patients with no IPVD or HPS.
- This finding in predominantly Child's class A cirrhotic argues the role of H2S in pathogenesis of HPS and needs further studies.

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S.No	Hosp No	name	residenc	GROUP	age	sex	Dyspnea	Clubbing	Cyanosis	Oxtahodeo	Spinever	CRAyest X	Hb	platelet	counts	TB	DB	protein	albumin	AST	ALT	Alkaline	phos	PT	INR	CREATINI	NE	CTP	MELD	pAO2	p(A-a)O2	ECHO CONTRAST	IPVD	H2S
1	613308D	WILLIAM	s	2	51	M	NO	NO	NO	NO	NO	NORMAL	7.1	75000		3.9	1.8	6.4	3.7	44	29	116		17.2	1.58			C	19	96	13.7	POSITIVE	25	48.93
2	883110D	PATNAM S RAO	s	2	40	M	YES	NO	YES	NO	NO	NORMAL	11	26000		18	16	4.3	1.9	90	38	55		19.2	1.91	1.29		C	27	96		POSITIVE		83
3	287217F	PACHIYAPAN	s	2	65	M	NO	NO	NO	NO	NO	NORMAL	6.8	174000		1.2	0.7	5.2	1.3	34	20	90		11	1.01			C	20	97	10.2	POSITIVE	25	47.14
4	460640C	RATNA KUMAR	s	2	38	M	NO	YES	NO	NO	NO	NORMAL	10	19000		2.6	1.3	6.7	3.4	64	36	115		16.8	1.52			C	16	113		POSITIVE	25	31.43
5	421799D	SAMPATH	s	1	69	M	NO	NO	NO	NO	NO	NORMAL	9.8	87000		0.5	0.2	5.9	2.4	16	2	44		11.8	1.08	2.58		B	16	110		NEGATIVE		25.71
6	667115C	GOPAL	s	2	55	M	NO	NO	NO	NO	NO	NORMAL	8.2	23000		2.5	1	6	2.5	90	59	74		15.8	1.45	0.9		C	12	100	13.4	POSITIVE	25	46.43
7	816337D	SHEFALI	s	1	55	F	NO	NO	NO	NO	NO	NORMAL	8.7	60000		0.7	0.3	6.9	2.8	30	20	83		11.4	1.05	0.88		A	7	98		NEGATIVE		0
8	350644F	BEER MD	s	1	32	M	NO	NO	NO	NO	NO	NORMAL	15	268000		1.2	0.5	7.8	4.7	33	20	95		14.3	1.3	1.03		A	10	97		NEGATIVE		8.46
9	352684F	SHIVA PRASAD	n	2	62	M	NO	NO	NO	NO	NO	NORMAL	11	38000		2.3	1.3	7.3	3.1	59	24	106		14.4	1.31	0.89		A	13	127		POSITIVE	10	10.77
10	289410F	SUNIL KR PRASAD	n	1	51	M	NO	NO	NO	NO	NO	NORMAL	11	128000		1.5	0.8	8.9	2.6	44	19	80		14.6	1.33	1.7		B	16	138		NEGATIVE		12.31
11	255130F	KISHORI SWARNAKAR	n	1	48	M	NO	NO	NO	NO	NO	NORMAL	11	105000		0.7	0.3	8.2	4.4	42	47	122		12.3	1.12	1		A	8	98		NEGATIVE		8.57
12	999215D	SELVI	s	1	26	F	NO	NO	NO	NO	NO	NORMAL	11	75000		1.4	0.3	9	4.8	51	39	227		12.5	1.14	0.92		A	9	105		NEGATIVE		5
13	402796F	PREMLAL	s	3	28	M	NO	NO	NO	NO	NO	NORMAL	9.1	24000		1.9	1.1	7.3	2.8	75	54	214		12.8	1.17	0.98		A	11	88.5	17.8	POSITIVE	25	56.43
14	245981F	SHANTI PADA CHAKRAVARTI	n	2	61	M	NO	NO	NO	NO	NO	NORMAL	12	55000		0.6	0.3	8.5	3.8	59	37	79		11.6	1.06	0.98		A	7	83		POSITIVE	25	7.14
15	359961F	SATHYAMOORTHY	s	1	63	M	NO	NO	NO	NO	NO	NORMAL	6.7	136000		0.5	0.2	5.4	3.2	21	8	66		13.6	1.24	1.24		B	11	99		NEGATIVE		8.57
16	360974F	SUBBA RATNAM	s	2	45	M	NO	NO	NO	NO	NO	NORMAL				0.6	0.4	6.8	3	68	32	131		12.1	1.11	1.07		A	8	107		POSITIVE	10	5.71
17	913094D	RENUGOPAL	s	2	74	M	NO	NO	NO	NO	NO	NORMAL	7	144000		1	0.6	5.6	2.2	66	52	118		13.2	1.2	1.4		C	14	115		POSITIVE	50	17.14

18	358396F	URMILA JHA	n	2	62	F	NO	NO	NO	NO	NO	NORMAL	11	123000	2.4	1.9	6.2	2.4	63	32	107	13.6	1.24	1.3	B	15	87		POSITIVE	25	5.71
19	361792F	SEKH BABAR ALI	n	1	51	M	NO	NO	NO	NO	NO	NORMAL	10	87000	0.4	0.2	7.5	3.4	38	15	145	11.5	1.05	1.02	A	7	96		NEGATIVE		
20	386379F	PRADEEP	n	1	52	M	NO	NO	NO	NO	NO	NORMAL	15	130000	2.3	0.6	7.5	4.6	79	117	103	11.5	1.05	1.21	A	12	101		NEGATIVE		15.71
21	417404F	RANJIT PATHAK	n	2	41	M	NO	NO	NO	NO	NO	NORMAL	7	193000	0.8	0.3	8.6	3.5	32	11	100	14.2	1.29	1.16	A	11	98		POSITIVE	25	16.43
22	424142F	LELLAPALLI VENKATA SUBBAIAH	s	3	27	M	NO	NO	NO	NO	NO	NORMAL	11	106000	3.1	1.5	8	3.9	56	22	129	13.6	1.24	0.8	A	13	79		POSITIVE	10	6.43
23	416260F	SAIBAL KUMAR DATTA	n	3	49	M	NO	NO	NO	NO	NO	NORMAL	14	81000	4	1.6	7.3	3.1	45	11	226	15.1	1.37	0.83	B	14	48		POSITIVE	50	64.28
24	330111F	KRISHNA SHARMA	n	2	36	M	NO	NO	NO	NO	NO	NORMAL	16	45000	3	0.9	7	3.4	102	85	99	15.8	1.43	0.88	B	15	94		POSITIVE	25	
25	400167D	RAMESH C	s	2	36	M	NO	NO	NO	NO	NO	NORMAL	6.1	53000	1.4	0.3	5.4	2.4	35	16	41	16.2	1.47	0.98	B	12	100		POSITIVE	25	37.86
26	301248F	LAKHO PATI DEVI	n	1	37	F	NO	NO	NO	NO	NO	NORMAL	12	52000	1.8	0.7	7.6	4.7	50	42	71	12	1.1	0.68	A	10	99		NEGATIVE		47.14
27	287608D	NEELAM MISHRA	n	2	42	F	NO	NO	NO	NO	NO	NORMAL	12	18000	1.8	0.6	7.7	3.9	34	17	129	11.4	1.04	0.67	A	9	89		POSITIVE	10	47.14
28	438995F	BIPLAB KANTI CHOWDHURI	n	3	35	M	NO	NO	NO	NO	NO	NORMAL	11	60000	1.7	0.7	6.9	4.4	44	19	181	13	1.19	0.85	A	10	88	16.9	POSITIVE	10	
29	324396F	USHA DEVI GUPTA	n	1	44	F	NO	NO	NO	NO	NO	NORMAL	9.4	106000	3.1	3	6.3	2.6	88	46	117	17.7	1.6	0.85	B	16	112		NEGATIVE		7.86
30	457494F	PRATIMA CHAKRABORTY	n	2	41	F	NO	NO	NO	NO	NO	NORMAL	8.6	99000	0.9	0.6	7.8	3.4	36	21	74	14	1.27	0.9	A	9	87		POSITIVE	25	20.71
31	282818F	RAKESH KUMAR GERARI	n	2	29	M	NO	NO	NO	NO	NO	NORMAL	12	43000	2.1	0.7	7.1	3.6	39	20	141	15.4	1.4	0.82	A	13	89		POSITIVE	25	26.1
32	049872D	ARUN KUMAR	n	2	38	M	NO	NO	NO	NO	NO	NORMAL	10	30000	3	0.6	6.1	2.7	34	19	83	16.7	1.51	0.7	B	15	89		POSITIVE	10	16.43
33	472273F	RAJESH SONAR	n	1	38	M	NO	NO	NO	NO	NO	NORMAL	9	54000	1.2	0.5	5	2.8	23	5	53	12.4	1.13	0.91	B	8	96		NEGATIVE		12.14
34	449735F	PARO DEVI	n	1	41	F	NO	NO	NO	NO	NO	NORMAL	9.8	105000	0.7	0.5	7.3	3	23	10	88	11.7	1.07	1.05	B	8	91	16	NEGATIVE		21.1
35	454856F	MD FAZAL P	n	3	24	M	NO	NO	NO	NO	NO	NORMAL	11	26000	5.5	3	6.6	2.9	52	12	109	15.6	1.42	1.16	B	18	58		POSITIVE	50	24.67
36	447117F	ANIL MUNDA	n	2	20	M	NO	NO	NO	NO	NO	NORMAL	14	122000	0.6	0.2	7.2	4	39	24	94	12.6	1.15	0.97	A	8	95		POSITIVE	10	30.71
37	433905F	ALAMELU	s	1	50	M	NO	NO	NO	NO	NO	NORMAL	11	77000	4.8	2.5	7.4	2.3	78	22	125	15.1	1.37	0.91	C	16	91		NEGATIVE		15.71
38	201250B	SANKARI V	s	2	42	F	NO	NO	NO	NO	NO	NORMAL	12	50000	1	0.8	6.7	3.1	40	11	217	13	1.19	0.69	B	8	95	11	POSITIVE	10	15

39	494961F	MD LOKMAN	n	1	40	M	NO	NO	NO	NO	NO	NORMAL	12	132000	4.9	0.5	7.5	4.4	56	43	103	11.6	1.06	0.98	B	13	57	48	NEGATIVE		21.43
40	379830F	ADEM SESHGIRI	s	3		M	NO	YES	NO	NO	NO	NORMAL	15	69000	3.4	0.9	7.6	4.6	28	17	85	13	1.18	0.94	A	13	66		positive	50	6.67
41	756857C	JEYALAKSHMI	s	3	43	F	YES	YES	NO	YES	NO	NORMAL	13	30000	3.4	1.4	6.2	2.4	59	8	120	17.4	1.56	0.8	B	17	78	40.6	POSITIVE	50	28.57
42	612267F	MATHEW	s	3	55	M	NO	NO	NO	NO	NO	NORMAL	12	74000	4.4	1.7	6	3	64	30	56	18.2	1.63	1.12	B	16	68		POSITIVE	25	
43	369432F	SHYAMAL KR SAHOO	n	1	52	M	NO	NO	NO	NO	NO	NORMAL	11	72000	1.9	0.8	8.3	4.1	43	47	62	12.4	1.13	0.89	A	10	98		NEGATIVE		
44	371206F	JAMUNA PRASAD	n	1	50	M	NO	NO	NO	NO	NO	NORMAL	14	46000	2	0.6	9.1	3.6	41	18	80	13.5	1.23	1.1	A	12	97		NEGATIVE		18.57
45	262086F	JHARESWAR NAYAK	n	1	57	M	NO	NO	NO	NO	NO	NORMAL	12	97000	0.5	0.2	8.2	4	27	13	70	11.1	1.02	1.08	A	7	110		NEGATIVE		14.29
46	323326f	SK DOLON	n	1	31	M	NO	NO	NO	NO	NO	NORMAL	12	85000	0.5	0.2	7.5	4	58	50	205	11.7	1.07	1.08	A	7	100		NEGATIVE		28.57
47	149839F	RABIN BAG	n	1	54	M	NO	NO	NO	NO	NO	NORMAL	14	68000	1.7	0.6	7.3	3.7	84	67	132	12.7	1.16	1.36	B	10	88	18	NEGATIVE		
48	633532F	SARITA DEVI	n	1	25	F	NO	NO	NO	NO	NO	NORMAL	7.5	36000	3.8	0.6	5.7	3.3	27	15	49	13.8	1.25	0.46	B	14	42		NEGATIVE		
49	681191F	SWAROOP	n	3	16	M	NO	YES	NO	N	YES	NORMAL	9.8		4.1			2.7	57	37	318	16.7	1.5	0.75	A	16	88	23	POSITIVE	50	
50	787345D	JOY JOSE	s	2	57	M	NO	YES	NO	N	N	NORMAL	9.7	66000	2.4	1.3	6.3	3	46	20	136	15.2	1.38	1.07	C	15	115	-2	POSITIVE	10	18.57
51	699746F	RAJENDRAN	s	3	56	M	NO	NO	NO	N	N	NORMAL	15	78000	3.4	1.4	6.2	2.6	58	37	133	15.4	1.39	1	C	15	73.7	44.9	P0SITIVE		
52	068105F	MATHEW VERGHESE	s	3	68	M	NO	N	NO	N	N	NORMAL	11	55000	3.5	1.5	6.1	2.2	59	21	121	24	2.12	0.69	C	20	59.2	52.2	POSITIVE	50	
53	706559F	SHAIK KHALEEL	n	3	36	M	NO	N	NO	N	N	NORMAL	16	22000	1.6	0.7	7.3	3.9	20	13	99	14.5	1.31	0.94	A	11	42		POSITIVE	50	14.29
54	494572F	BIRENDRA YADAV	n	2	31	M	NO	N	NO	N	N	NORMAL	13	48000	3.1	0.5	6.9	4.1	33	25	53	14.2	1.29	0.83	A	13	97		POSITIVE	10	10.83
55	648000F	RAJESH PANDEY	n	3	40	M	NO	N	NO	N	N	NORMAL	7.8	38000	0.7	0.4	6.5	2.4	50	41	128	14.7	1.33	0.84	A	10	83		POSITIVE	10	11.43
56	093521F	SANCHITA	n	1	48	F	NO	N	NO	N	N	NORMAL	12	52000	1.8	0.8	7.9	4.5	44	12	130	14.9	1.36	1.04	A	12	95		NEGATIVE		6.67
57	302051D	MANOJ KUMAR	n	1	38	M	NO	N	NO	N	N	NORMAL	14	127000	2	0.8	6.8	3.4	49	38	108	12.9	1.16	0.85	A	11	46		NEGATIVE		0
58	064554F	NURUJEN	s	1	47	F	NO	N	NO	N	N	NORMAL	9.7	86000	0.3	0.1	6	3.4	18	9	88	11.9	1.09	0.92	A	7	86		NEGATIVE		